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Residues that affect human Argonaute2 concentration in cytoplasmic processing bodies

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ABSTRACT

Sequence-specific gene silencing triggered by double-stranded RNA is a fundamental gene regulatory mechanism present in almost all eukaryotes. Argonaute2 (Ago2) is the central protein component of RNA-induced silencing complex (RISC), and resides in cytoplasmic processing bodies (P-bodies). In the present study, we demonstrated one human mutant Ago2 protein containing 6 point mutations (G32W, F128L, R196Q, P458S, T741A, S752G) failed to accumulate in P-bodies. Analysis of the different Ago2 revertants indicates the S752 as a key amino acid for P-body localization of Ago2. The S752 is evolutionary conserved in the Piwi domain of Ago2 homologs from worms, insects, plants and mammals. We further showed the single point mutation S752G interfering the interaction between Ago2 and Dcp1a, a key component of P-bodies.

Programmed mRNA turnover plays an essential role in regulating gene expression [1]. The major pathway of eukaryotic mRNA decay is initiated with the deadenylation and followed with decapping by Dcp1/Dcp2 complex [2]. Decapping and degradation of mRNAs occur in P-bodies [3–5]. RNA interference (RNAi) was first discovered in the nematode worm *Caenorhabditis elegans* in response to double-stranded RNA [6]. Double-stranded RNAs are recognized by the RNAse III enzyme, Dicer, which cleaves the double-stranded RNAs into double-stranded small interfering RNAs (siRNAs) of 19–21 base pairs [6]. These siRNAs are recognized by RNA-induced silencing complex (RISC), which unwinds the double-stranded siRNAs and guides the siRNA to its target mRNA. The target mRNA cleaved by the endonuclease Ago2 results in sequence-specific gene silencing [7–11]. Ago2 is found to reside primarily in P-bodies and its location facilitates the siRNA-induced mRNA cleavage by the exonucleases and decapping enzymes [12,13].

Cells are rarely exposed to siRNAs under natural conditions except viral infections [14]. However the cleavage or translational repression of target mRNAs by microRNAs (miRNAs) may become a common phenomenon in all eukaryotes [12]. miRNAs are a large family of endogenous, small regulatory RNAs. The nascent transcripts of miRNA genes, pri-miRNAs, are cleaved by Drosha RNase III endonuclease in nuclei, which define one end of the mature miRNA. The other end is processed in cytoplasm by Dicer. Following maturation, miRNA pathway appears to be biochemically indistinguishable from that of RNAi. A single strand of the miRNA duplex is incorporated into RISC and directs the RISC to the target genes. Ago2 is a component of the miRNA-induced translational repression complex (miRNP), a subtype of RISC. Ago2/miRNP may direct miRNA-targeted mRNAs to P-bodies to prevent their translation [13–19]. Ago2 has also been shown to mediate the degradation of AU-rich mRNA through an miRNA-dependent mechanism [20].

Ago2 is a member of evolutionary conserved Argonaute protein family which constitutes proteins involved in a variety of RNA silencing [21–24]. Ago proteins contain an N-terminal PAZ domain and a C-terminal PIWI domain [22]. The PAZ domain is a novel RNA binding module that specifically recognizes the 3′ overhang of siRNA duplexes [25,26]. The PAZ domain has also been shown to be critical for miRNAs binding and P-body localization [27]. Liu et al generated two Ago2 mutant proteins, Ago2-PAZ9 and Ago2-PAZ10 containing 9 and 10 point mutations, respectively, within the PAZ domain. Both Ago2-PAZ9 and Ago2-PAZ10 retained the ability to interact with Dcp1a and Dcp2 but failed to accumulate in P-bodies [27]. Ago2 is the mediator of small RNA-guided gene-silencing pathways and localizes to cytoplasmic P-bodies, but there is little known about how Ago2 is concentrated in P-bodies. We, here report one human mutant Ago2 protein containing 6 point mutations (G32W, F128L, R196Q, P458S, T741A, S752G) also failed to accumulate in P-bodies. We called it Ago2 with 6 mutations (Ago2-6mut). We produced different revertants of Ago2-6mut by site-directed mutagenesis and examined the efficiency of the revertants localizing in P-bodies. The S752 in the PIWI domain

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appears to be a key amino acid for Ago2 accumulation in P-bodies and the interaction between Ago2 and Dcp1a.

Materials and methods

DNA constructs. Human Ago2 was cloned from HeLa cells using RT-PCR and Ago2 coding region was subcloned into vector pcDNAM with Myc-tag at 3′ end. Ago2 mutants were introduced by site-directed mutagenesis using the QuickChange Kit from Stratagene (La Jolla, CA). All plasmids were sequenced by Invitrogen (Shanghai) and the resulting recombinant plasmid had the desired mutation(s). GFP-Dcp1a was as previously described [13].

Cell culture and transfection. HeLa and HEK293T cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 ug/ml penicillin and streptomycin in a 37 °C incubator with 5% CO_2. Cells were transiently transfected with 2 μg (total) of plasmid DNA per well in 6-well plate using Lipofectamine 2000 (Invitrogen).

Immunofluorescence. HeLa Cells cultured on coverslips were fixed in PBS with 4% paraformaldehyde for 10 min at room temperature, permeabilized in PBS containing 0.2% Triton-X100 for 5 min, then blocked with 5% BSA for 1 h. Cells were incubated with monoclonal anti-Myc antibodies (Santa Cruz Biotechnology) for 1 h, rinsed, and then incubated with Rhodamine-Red-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology). The samples were mounted and examined under fluorescent microscope.

Electrophoresis, Western blot, and immunoprecipitation analysis. Total cell lysates were prepared as previously described for SDS–PAGE and isoelectric focusing electrophoresis (IEF), respectively [20,28]. For co-immunoprecipitation, cell lysates prepared as above were incubated with anti-Myc beads (Sigma) and gently shaken for 4 h at 4 °C. The beads were washed three times with the lysis buffer. Then, 50 ul SDS sample buffer was added, and the samples were heated for 5 min at 100 °C. The supernatants were applied to SDS gel and detected by immunoblotting.

Results and discussion

During the investigation of miRNA mediated AU-rich mRNA decay, we cloned human Ago2 from HeLa cells using RT-PCR [20]. Interestingly, we found one mutant Ago2 protein, Ago2-6mut, containing 6 point mutations (G32W, F128L, R196Q, P458S, T741A,

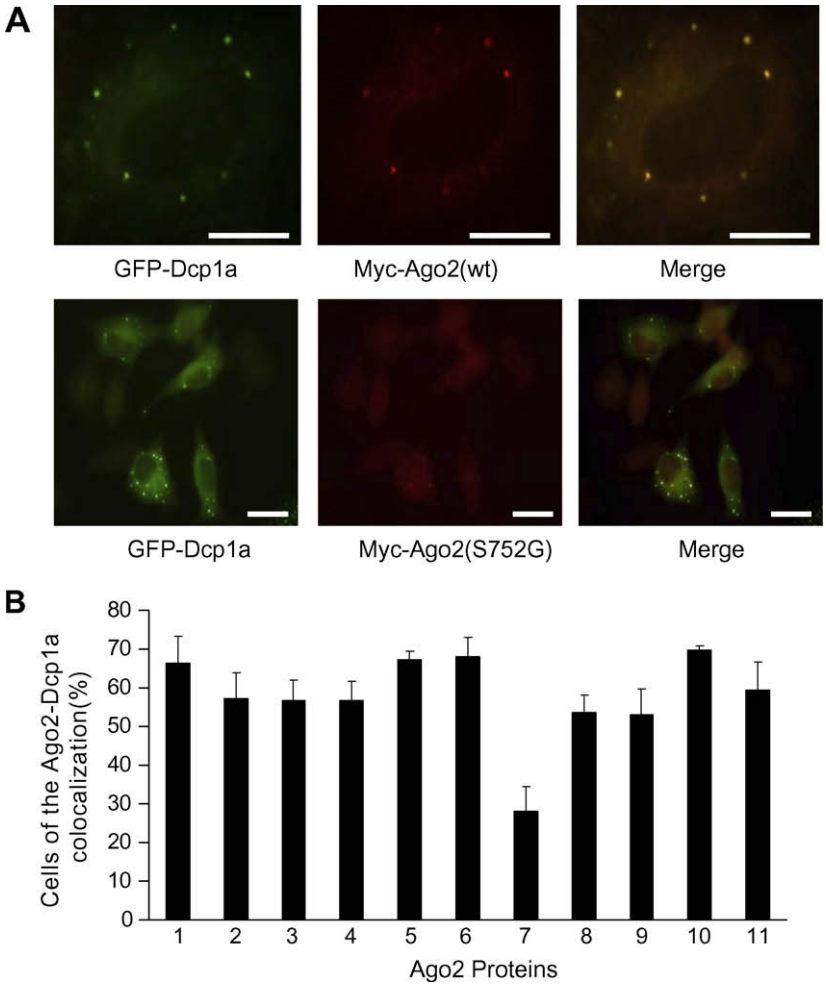

Fig. 1. The colocalization of wild-type or mutant Ago2 proteins with Dcp1a in HeLa cells. Myc-tagged wild-type (wt) or mutant Ago2 proteins were co-expressed with GFP-tagged Dcp1a in HeLa cells. Ago2 proteins localized to discrete cytoplasmic foci(P-bodies) by staining with primary mouse anti-Myc antibodies and secondary Rhodamine Red-conjugated goat anti-mouse IgG antibodies. Dcp1a was visualized by GFP. (A) Images of Myc-Ago2 and GFP-Dcp1 in red and green, respectively; scale bars represent 10 μm. (B) Co-localization rates of the Ago2 proteins with Dcp1a were calculated and compared. 1, Myc-Ago2(wt); 2, Myc-Ago2(G32W); 3, Myc-Ago2(F128L); 4, Myc-Ago2(R196Q); 5, Myc-Ago2(P458S); 6, Myc-Ago2(T741A); 7, Myc-Ago2(S752G); 8, Myc-Ago2(G32W, F128L); 9, Myc-Ago2(G32W, F128L, P458S); 10, Myc-Ago2(G32W, F128L, R196Q, P458S); 11, Myc-Ago2(G32W, F128L, R196Q, P458S, T741A). In random areas, the cells with GFP discrete foci were selected, the co-localization rate of GFP-Dcp1a and Myc-Ago2 labeled in Red was calculated. More than 500 cells were counted for every experiment and three separate experiments were carried out. Error bars represent standard deviation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

S752G) failed to accumulate in P-bodies. To elucidate which mutation or mutations caused the defects of Ago2 in the P-body localization, we created a series of Ago2 mutants through site-specific mutagenesis. The resultant plasmids were cotransfected into HeLa cells with GFP-Dcp1a. GFP-Dcp1a chimeric protein was selected as a marker of P-bodies [13,27]. The colocalization of the Ago2 proteins and GFP-Dcp1a represented the accumulation of Ago2 in P-bodies and was visualized by immunofluorescence staining. We first tested Ago2 with all the 6 single point mutations, respectively, and determined the colocalization rate by counting 500 randomly picked up cells. We observed that the accumulation of Ago2-S752G in P-bodies significantly decreased ($p < 0.01$), while Ago2-G32W, Ago2-F128L, and Ago2-R196Q showed slightly reduced colocalization rate with GFP-Dcp1a (Fig. 1A and B). The S752 appeared to be a key amino acid for Ago2 accumulation in P-bodies. Then we investigated Ago2 with different combinations of other 5 mutations (G32W, F128L, R196Q, P458S, T741A) and all the mutant Ago2 proteins including the one with all five mutations showed slightly lowered localization in P-bodies (columns 7–11 in Fig. 1 B). Therefore, we concluded that S752 is a critical residue for Ago2 localizing in P-bodies.

Since Ago2-S752G still can accumulate in P-bodies even though at very low level, we generated a series of Ago2 mutants all contained S752G and checked their localization in P-bodies. Addition of all the other point mutations except R196Q on Ago2-S752G did further decrease the localization rate of the Ago2 proteins in P-bodies (Fig. 2A and B). Moreover we observed that Ago2 with the 4 point mutations (G32W, F128L, P458S, S752G) completely abolished in P-bodies (Fig. 2A and B). We wondered whether these mutations changed Ago2 expression level or the protein stability. To exclude these possibilities we compared the protein level of the Ago2 mutants mentioned above with that of wild-type Ago2. Western blot assays showed that the protein levels of the Ago2 mutants were comparable with that of wild-type Ago2 (Fig. 3A). Taken together, our results suggest that the mutations we generated probably changed the conformation of Ago2 and the conformational change suppressed Ago2 localizing to P-bodies.

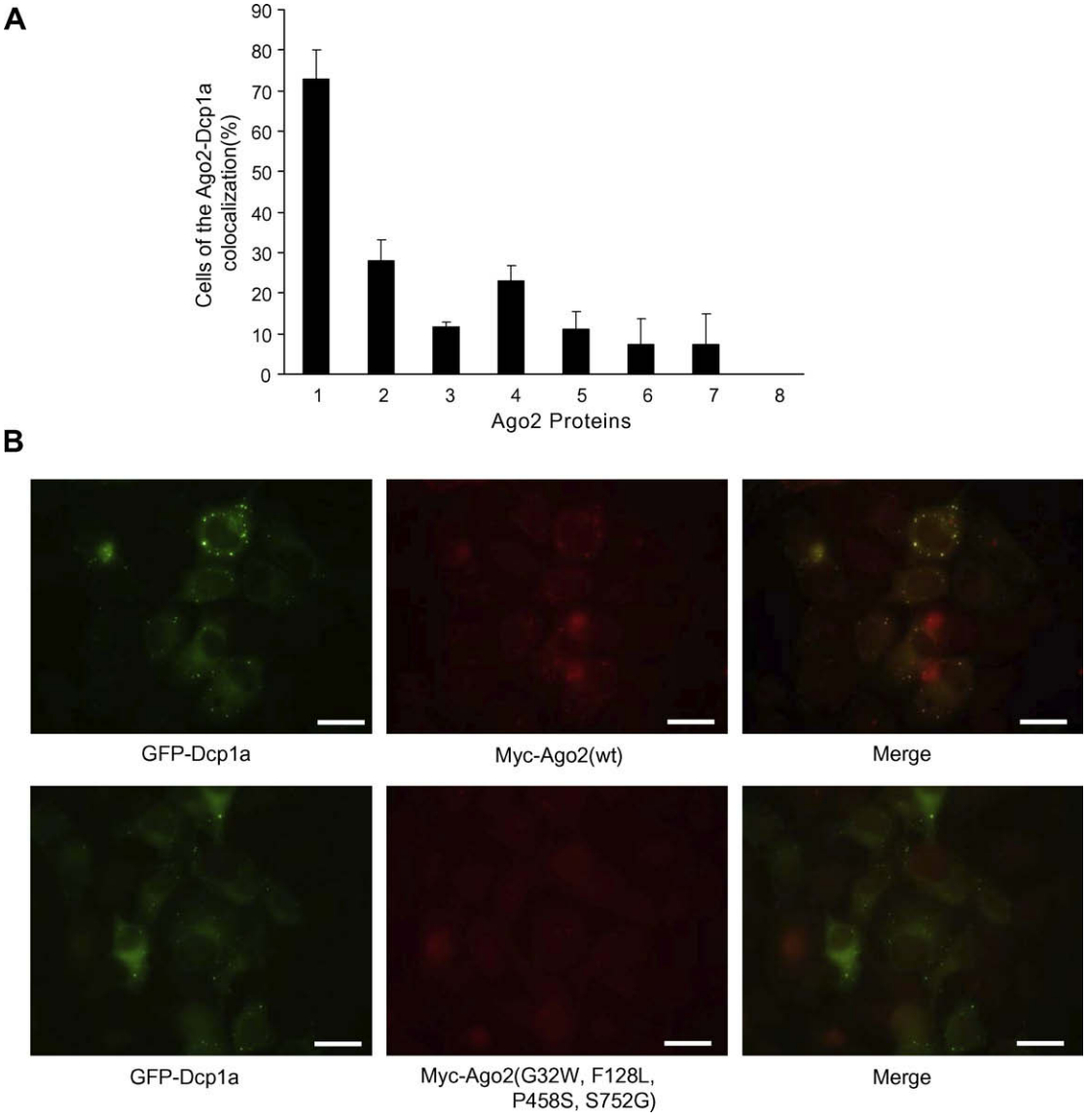

Fig. 2. The co-localization of GFP-Dcp1a with wt or mutant Myc-Ago2 containing S752G mutation and other mutations. Same standards were applied as in Fig. 1. (A) Histograms indicate co-localization rates of GFP-Dcp1a with 1, Myc-Ago2(wt); 2, Myc-Ago2(S752G); 3, Myc-Ago2(F128L, S752G); 4, Myc-Ago2(R196Q, S752G); 5, Myc-Ago2(P458S, S752G); 6, Myc-Ago2(T741A, S752G); 7, Myc-Ago2(G32W, T741A, S752G); 8, Myc-Ago2(G32W, F128L, P458S, S752G). Error bars represent standard deviation. (B) Ago2 with four mutations (G32W, F128L, P458S, S752G) failed to accumulate in P-bodies. Scale bars represent 15 μm.

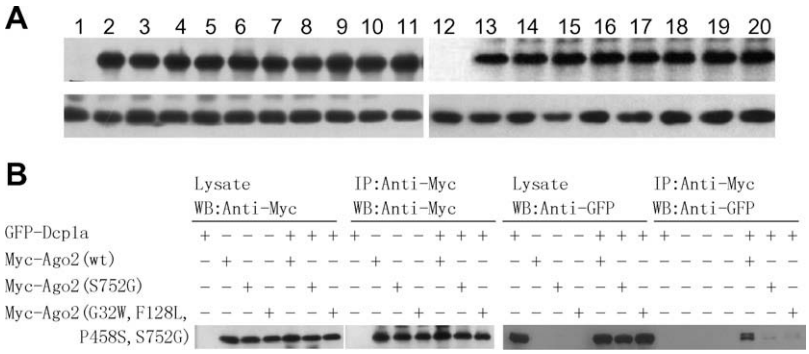

Fig. 3. The mutant Ago2 proteins expressed at the similar level as wild-type Ago2 and the S752G mutation dramatically reduced the interaction between Ago2 and Dcp1a. (A) The upper panels show Myc-Ago2 protein expression levels analyzed by Western blotting with anti-Myc antibodies 24 h after transfection. 1, control; 2, Myc-Ago2(wt); 3, Myc-Ago2(G32W); 4, Myc-Ago2(F128L); 5, Myc-Ago2(R196Q); 6, Myc-Ago2(P458S); 7, Myc-Ago2(T741A); 8, Myc-Ago2(G32W, F128L); 9, Myc-Ago2(G32W, F128L, P458S); 10, Myc-Ago2(G32W, F128L, R196Q, P458S); 11, Myc-Ago2(G32W, F128L, R196Q, P458S, T741A); 12, control; 13, Myc-Ago2(wt); 14, Myc-Ago2(S752G); 15, Myc-Ago2(F128L, S752G); 16, Myc-Ago2(R196Q, S752G); 17, Myc-Ago2(P458S, S752G); 18, Myc-Ago2(T741A, S752G); 19, Myc-Ago2(G32W, T741A, S752G); 20, Myc-Ago2(G32W, F128L, P458S, S752G). The lower panels show loading controls blotted with anti-α tubulin antibodies. (B) The interaction between Ago2 and Dcp1a.was shown with co-immunoprecipitation. GFP-Dcp1a with wt or mutant Ago2 co-expressed in HEK 293T cells. The cells were harvested 24 h after transfection. One-third of the cell lysates was analyzed by Western blotting with anti-Myc and anti-GFP antibodies. The rest of the cell lysates was subjected to immunoprecipitation with anti-Myc antibodies, and further analyzed by western blotting with anti-Myc and anti-GFP antibodies as indicated.

Ago2 was not only concentrated in P-bodies but also physically interacts with Dcp1a [27]. We asked whether the mutations involved in Ago2 P-body localization also affect the physical interaction between Ago2 and Dcp1a. Dcp1a was co-expressed with Ago2(wt), Myc-Ago2(S752G) or Myc-Ago2(G32W, F128L, P458S, S752G) in HEK 293T cells. The interactions between Dcp1a and different versions of Ago2 were evaluated by co-immnoprecipitation and Western blot. As shown in Fig. 3B, the single mutation S752G and 4 mutations (G32W, F128L, P458S, S752G) of Ago2 drastically decreased the interaction between Ago2 and Dcp1a. It suggests that S752 of Ago2 is essential in Ago2-Dcp1a interaction. It was shown previously that Dcp-Ago interactions were resistant to RNaseA and occurred even with non-P-body localized Ago2 containing mutations in that PAZ domain [27]. However RNase eliminates Ago2 localization in P-bodies [12]. Considered together, it indicates that the PIWI domain but not the PAZ domain may be required for Ago2-Dcp1a interaction and such interaction facilitates Ago2 to localize to P-bodies. Binding to RNAs is not essential for Ago2-Dcp1a interaction but may be important for Ago2 localizing to P-bodies.

Recently, Ago2 was shown to be phosphorylated by p38 MAPK pathway and the phosphorylation of S387 facilitated Ago2 localization in P-bodies [29]. The S387A mutation also reduced the localization of Ago2 to P-bodies. We performed multiple alignments of Ago2 homologs from different species using online ClustalW program (http://www.ch.embnet.org/software/ClustalW.html). The S387 only exists at the corresponding position in mammalian Ago2 proteins, whereas S752 is evolutionary conserved in the Piwi domain of Ago2 homologs from worm, insects, plants and mammals (Fig. S1). We speculated that the S752 might also be a phosphorylation site. Wild-type Ago2 and Ago2-S752G or the Ago2 containing 4 point mutations (G32W, F128L, P458S, S752G) were transiently expressed in HEK293T cells. The cells were treated with p38 inhibitor SB203580 prior stimulated with Arsenite. Whole cell lysates were subjected to IEF assay followed by Western blotting to check the phosphorylation status of the Ago2 proteins. The mutant Ago2 proteins migrated normally as wild-type Ago2 in IEF assays (Fig. S2). S752 appears unlikely as a phosphorylation site of Ago2. Ago2 protein sequence was analyzed with online program Netphos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) [30]. In consistent to the experimental data, S387 appears as a weak phosphorylation site while S752 as a non-phosphorylation site (Table. S1).

In an advance online published structure of Ago2, S752 has been shown to be one of the residues involved in pivot motions between PAZ and PIWI domains. This closing movement may generate a narrower nucleic acid binding channel which is necessary to hold and orient the bound small RNAs [31] (http://www.nature.com/nature/journal/vaop/ncurrent/abs/nature07315.html). Several observations in this work indicate that the S752 is critical for the localization of Ago2 in P-bodies and essential for the interaction between Argonaute2 and Dcp1a. S752 is highly conserved during evolution and unlikely to be a phosphorylation site of Ago2.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.11.098.

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